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We demonstrated that Venezuelan equine encephalomyelitis (VEE) virus replicated in and adapted rapidly to human diploid cell strain WI-38. Peak titers of approximately $10^{9.8}$ mouse intracerebral 50% lethal doses were obtained at low passage levels in Eagles basal medium supplemented with calf serum. VEE virus replicated poorly in serum-free medium. Propagation of VEE virus was accompanied by the production of hemagglutinin and cytopathogenic effects.

Venezuelan equine encephalomyelitis (VEE) virus grows in Maitland-type chick embryo tissue (7), in tissue cultures of Chang's human liver and conjunctiva, monkey kidney, chicken embryo, embryo mouse lung, guinea pig kidney, L cells (2), and HeLa cells (8).

This report describes the growth of VEE in human diploid cell strain WI-38, a cell strain derived from the lungs of a human embryo. This cell strain has a limited life span of about 50 generations and is free of known contaminating viruses (5).

Diploid cell strains have supported the replication of human and animal viruses (5, 6, 14). It was of interest to us to investigate the ability of WI-38 cells to support the growth of VEE virus as a possible method for the production of virus for the preparation of inactivated vaccines.

The Trinidad donkey brain strain of VEE virus (2) was acquired at this laboratory as a 10% chick embryo suspension in beef heart infusion broth (BHIB). A working seed virus was prepared from the 14th egg passage in 10-day embryonated eggs. The seed virus was a 15% embryo suspension in BHIB and contained $10^{10.9}$ mouse intracerebral 50% lethal doses ($MICLD_{50}$)/ml.

Human diploid cell strain WI-38 (4) was grown as monolayers in glass or plastic flasks. Monolayers also were obtained from Flow Laboratories, Rockville, Md. Cell cultures were fed with Eagles basal medium (BME) (1) with Hanks salts (HBME), supplemented with 10% fetal calf serum (FCS). Antibiotics were added to the medium to give final concentrations of either 100 units of penicillin plus 100 μg of streptomycin or 50 μg of aureomycin per ml.

Monolayers were washed and inoculated with various dilutions of VEE virus as described below. After adsorption for 45 min at 35 C, the inoculum was removed, and the cell sheet was washed in accordance with the experimental design. The cultures then were fed BME with Hanks or Earles salts plus 3% FCS and incubated at 35 C for designated periods of time. The infected culture fluid was harvested, clarified by low-speed centrifugation (700 $\times$ g) at 4 C, dispensed into serum bottles, and stored in a mechanical freezer at -70 C until assayed.

Infectivity titrations were made by intraperitoneal (IP) and intracerebral (IC) inoculation of Swiss mice, and the LD_{50} end points were calculated by the method of Reed and Muench (11). Culture fluids were tested for the presence of hemagglutinin (HA), by the microtiter technique (13) using goose erythrocytes as described previously (12).

Preliminary attempts to propagate VEE virus in WI-38 cells revealed the presence of a toxic factor for diploid cells in the virus egg seed. The toxic factor was eliminated by diluting the seed out to 10^{-6}. When monolayers were inoculated with a multiplicity of 0.002 $MIPLD_{50}$ of VEE virus per cell, very little demonstrable virus was present at 18 hr and the titer rose to a peak of $10^{9.0}$ mouse IP LD_{50} ($MIPLD_{50}$)/ml at 42 hr and then decreased to $10^{6.6}$ $MIPLD_{50}$/ml at 96 hr. A 1+ cytopathogenic effect (CPE) was present at 18 hr and increased to complete destruction of the monolayer at 96 hr. HA was present in titers of 1:40 at 42 and 72 hr, but was not demonstrable at 18 and 96 hr.

Monolayers were washed three times with saline A to remove residual serum and then inoculated with serial 10-fold dilutions of VEE virus in phosphate-buffered saline (PBS). The virus had previously undergone one passage in

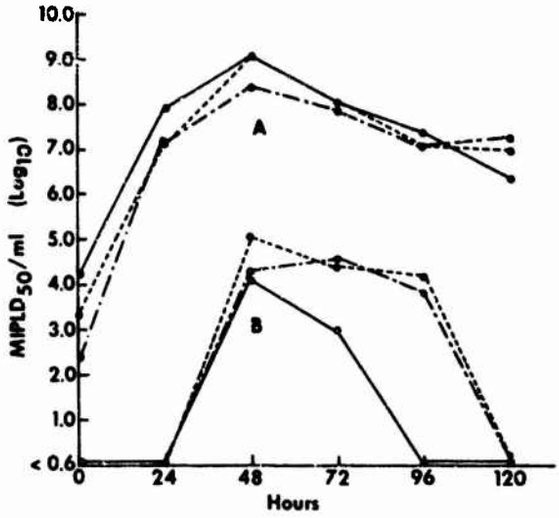

FIG. 1. *Effect of inoculum concentration and serum on growth of VEE virus in WI-38 cells. (A) EBME medium supplemented with 3% FCS, (B) serum-free medium. Symbols: ——, 10^{-1}; – – –, 10^{-2}; – –, 10^{-3} dilutions of inoculum.*

WI-38 cells. After a 45-min adsorption period at 35 C, the nonadsorbed virus was removed, and the cell sheets were washed twice with BME with Earles salts (EBME). The infected monolayers then were fed maintenance medium consisting of EBME with and without 3% FCS. Samples of the culture fluid were taken at this time and at 24-hr intervals. Figure 1 shows the virus titers obtained after incubation at 35 C. The results indicate that FCS is required for propagation of VEE in WI-38 cells. In the presence of serum, the titer rose sharply at 24 hr, with a peak at 48 hr followed by a gradual decrease in titer through 120 hr of incubation. Calf serum also appears to protect the virus on the cell sheet since in the absence of serum no virus could be detected by mouse inoculation at zero time. At this time virus titers as high as 4.2 $MIPLD_{50}$/ml were present in the culture fluids supplemented with serum. Titers in the culture fluids of monolayers maintained on serum-free medium did not exceed 5.1 $MIPLD_{50}$/ml. Identical peak titers ($10^{9.1}$ $MIPLD_{50}$/ml) were obtained in serum containing medium inoculated with 10^{-1} or 10^{-2} dilutions of virus. However, the titer in the flask inoculated with a 10^{-3} dilution was 0.7 log lower.

Passage of VEE in WI-38 cells resulted in rapid adaptation. Five passages were done in monolayers maintained with HBME supplemented with 3% FCS. When undiluted 48-hr-old infected culture fluids were transferred into fresh monolayers, high titers (10^{8} to 10^{9} $MICLD_{50}$/ml) were obtained on initial passage. Peak titers as high as $10^{9.8}$ $MICLD_{50}$/ml were obtained as early as the second passage. HA titers ranged from <1:2 to 1:128 per 0.05 ml. Passage of VEE virus in WI-38 did not enhance the production of HA, and, in some passages, HA could not be detected, although the mouse titer was approximately 10^{8} $MICLD_{50}$/ml.

Human diploid cell strain WI-38 appears to be an excellent substrate for the propagation of VEE virus. The data indicate that the yield of VEE virus is affected by the presence of FCS in the maintenance medium. Thus, there was little virus yield in monolayers maintained in serum-free medium, but high yields were obtained with the addition of serum. Similar results have been reported by Plotkin et al. (9) with rubella virus in WI-38 cells. Low yields obtained in serum-free cultures may be due to instability of VEE virus. Hardy and Brown (3) reported that VEE virus was less stable in medium 199 at 37 C than in the same medium without serum.

VEE virus appears to propagate more slowly in WI-38 cells than in L or chick fibroblast cells. Peak titers were observed at 48 hr of incubation in WI-38 cells, but have been reported to appear 24 hr earlier in L (3) and in chick fibroblast cells (15). It is noteworthy that although peak titers appeared after 48 hr of incubation in WI-38, maximal CPE occurred 48 hr later. Hardy and Brown (3) found that CPE appeared 12 to 24 hr after peak titers were reached in L-cell monolayers.

Passage of VEE virus through WI-38 did not increase the quantity of HA, and, in some experiments, the ability to produce this antigen was lost after a few successive passages, although the infective titer for mice remained at a high level. Similar observations were reported by Yershov and Vagzhanova (15) in chick fibroblast monolayers.

Since the antigenic capacity of inactivated virus vaccines is directly related to antigenic mass (10), the production of high titers of VEE virus in human diploid cell strain WI-38 suggests that this cell strain might be an appropriate source for the preparation of an inactivated vaccine.

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